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Protein uptake disturbs collagen homeostasis in proximal tubule-derived cells

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Protein uptake disturbs collagen homeostasis in proximal tubule-derived cells.

Background. Interstitial fibrosis is of major importance for the deterioration of renal function, leading to uremia. Interaction of filtered proteins with proximal tubular cells is important for the onset and development of tubulointerstitial damage.

Methods. We investigated the effects of protein endocytosis on collagen homeostasis and signaling pathways of proximal tubule-derived cells (OK cells, LLC-PK_1 cells), which express the endocytic machinery typical for the proximal tubule (megalin and cubilin), and compared it to renal epithelial cells with low endocytic activity (MDCK, IHKE1, NHE3-deficient OK cells). Collagen homeostasis was assessed by proline incorporation, ELISA, and Western blot. Matrix metalloproteinase (MMP) activity was assessed by gelatinase assay. Signaling pathways were monitored by reporter gene assay.

Results. Albumin, glycated albumin, fatty acid-free albumin, or globulins led to an increase of secreted collagen (types I, III, and IV) in OK and LLC-PK1 cells. In cells with low protein uptake activity, albumin exposure inhibited collagen secretion. Western blot analysis showed an increase of cellular collagen. MMP activity was significantly decreased by albumin exposure. Furthermore, albumin exposure led to activation of the NF-kB-, AP1-, NFAT-, SRE-, and CRE-pathways. Inhibition of NF-κB, PKC, or PKA partially reversed the effects of albumin. In addition, inhibition of albumin endocytosis reduced collagen secretion and activation of the signaling pathways.

Discussion. The data show that endocytic uptake of proteins disturbs collagen homeostasis in proximal tubular cells. This disturbed matrix homeostasis probably supports the progression of interstitial fibrosis, which is of importance for the development of renal insufficiency.

Renal proximal tubular protein reabsorption is of major importance because it prevents the loss of vitamins, hormones, and amino acids [1, 2], but at the same time enhanced tubular protein loads can induce tubulointerstitial inflammation and fibrosis [3, 4] and change proximal tubular gene expression [5]. One of the major receptors for proximal tubular protein endocytosis is the megalin/cubilin complex [1, 6], which accepts a variety of other ligands [1] and is responsible for the reabsorption of vitamin-binding proteins, hormone-binding proteins, hormones, and light chains.

The precise mechanisms leading to protein-induced inflammation or fibrosis are not yet completely understood but seem to involve the expression of pathophysiologically-relevant mediators (e.g., RANTES, MCP-1, NF-κB, AP1, and other mitogenic pathways in proximal tubular cells [3, 7–9]. The importance of protein uptake (i.e., receptor-mediated endocytosis) in this event has not been determined systematically. Furthermore, the effect of protein endocytosis on proximal tubular cell collagen homeostasis has not been studied at the cellular level. Besides its pathogenetic role in kidney diseases, renal collagen homeostasis has also attracted attention as a marker for the progression of kidney disease [10]. The proximal-tubule-derived opossum kidney (OK) cell line has been shown to be a suitable model system in order to study megalin/cubilin-mediated, clathrin-dependent endocytosis [11]. Because the amount of protein taken up is probably of crucial importance for the pathophysiologic outcome, we primarily used the OK cell line in order to determine the potential impact of albumin endocytosis on collagen homeostasis and the activation of signaling pathways in the present study.

METHODS

Materials

Media and fetal calf serum were obtained from Biochrom (Berlin, Germany). All other applied chemicals were obtained from Sigma (Deisenhofen, Germany), if not stated otherwise. Opossum kidney (OK) wild type cells were kindly provided by Dr. Biber, Department of Physiology, Zürich, Switzerland. NHE3-deficient OK cells, with dramatically reduced endocytic activity, have been characterized extensively [12]. LLC-PK1-, MDCK-, and IHKE1-cells were cultivated as described previously [11]. Prior to incubation with experimental media, cells

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were cultivated in serum-free media for 24 hours. Cell number and cell size were determined using a Coulter Counter Z2 series.

Determination of collagen secretion by collagenase-sensitive proline incorporation

Total collagen secretion and total secreted protein were assessed by [^{3}H]proline incorporation assay as described elsewhere [13, 14].

Determination of collagen secretion by ELISA

Media and collagen standards (Sigma, Deisenhofen, Germany) were incubated for 24 hours in 96-well Nunc-Immuno Maxisorb plates (Nalge Nunc International, Naperville, IL, USA), followed by washing and blocking with 2% bovine serum albumin in phosphate buffered saline. Subsequently, the wells were incubated with rabbit antibody against collagen I, collagen III, or collagen IV (1:1000; Biotrend, Köln, Germany). After three washes, HRP-conjugated secondary antibody (1:5000; Biotrend, Köln, Germany) was applied for one hour at room temperature. After three further washes, the wells were incubated with o-phenlyenediamine, and the reaction was stopped after 15 min with 1 N H_2SO_4. The absorbance at 490 nm was determined using a multiwell-multilabel reader.

Determination of cellular collagen by Western blot

Cells were lysed in ice-cold RIPA buffer and the lysates were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Subsequently, membranes were blotted with either a rabbit anticollagen type I, III, or IV antibody (1:1000, Biotrend, Cologne, Germany). The primary antibody was detected using horseradish peroxidase-conjugated secondary IgG (1:25,000; visualized by Amersham Corporation, ECL system. Linearity of the signal has been verified by serial dilution as recommended by the manufacturer. Densitometric analysis was performed using Sigmagel 1.05 software (Jandel, Corte Madera, CA, USA).

Determination of gelatinase activity

Gelatinase activity in cell culture media was determined by the ENZ®Gelatinase Assay Kit from Molecular Probes (Leiden, The Netherlands) using fluorescein-conjugated gelatin. The increase in fluorescence is a direct measure for gelatinase activity.

Reporter gene assay

Cell signaling was assessed by the Mercury™ Pathway Profiling reporter gene assay system from Clontech, Inc. (San Francisco, CA, USA) using secretory alkaline phosphatase (SEAP) as reporter, and pEGFP-C1 (Clontech, Inc., San Francisco, CA) as transfection efficiency control. SEAP activity in the media was determined with the AttoPhos® System from Promega (Mannheim, Germany), and the cellular EGFP content was determined using a microplate spectrofluorometer (Victor^2; Wallac, Turku, Finland). SEAP activity is always expressed as activity corrected for transfection efficiency, as determined by EGFP.

Calculations and statistics

Data are presented as mean values ± SEM. N represents the number of petri dishes. Cells of at least three passages were used for each experimental series. Significance of difference was tested by the Student *t* test, or ANOVA, as appropriate. Differences were considered significant if $P < 0.05$.

RESULTS

Determination of collagen secretion by collagenase-sensitive proline incorporation

As shown in Figure 1A, exposure to 100 mg/L of albumin for 48 hours significantly enhanced the collagen fraction in secreted protein, indicating that the effect was not simply due to an enhanced overall protein secretion. At 1000 mg/L of albumin, the collagen fraction decreased slightly as compared to 100 mg/L of albumin, indicating a biphasic dose-response curve. The overall secretion of protein (determined as trichloroacetic acid [TCA]-insoluble secreted radioactivity) was not reduced by exposure to albumin. Qualitatively similar effects were observed after 24 hours.

Determination of collagen secretion by ELISA

Figure 1B shows that exposure to albumin (OK cells, 48 hours) leads to a dose-dependent biphasic increase of collagen I, III, and IV secretion. When the concentration of albumin was enhanced above 1000 mg/L, we even observed a decrease of collagen I and III secretion below control values. Collagen secretion was also enhanced when the cells were exposed to γ-globulins (10 mg/L), but not during exposure to 100 mg/L 70 kD dextran (Fig. 1 C and D). Dextran is taken up by fluid-phase endocytosis, as described previously [2]. Glycated albumin (100 mg/L), or fatty-acid-free albumin (100 mg/L), exerted similar effects as compared with 100 mg/L of albumin (data not shown). Cell number or cell size was not enhanced significantly by exposure to albumin in concentrations up to 10,000 mg/L (Fig. 1E).

Determination of cellular collagen by Western blot

As shown in Figures 2A and B, the cellular levels of collagen I, III, and IV demonstrated a moderate increase during exposure to 100 mg/L of albumin. Furthermore, at 1000 mg/L, cellular collagen levels were decreased. This behavior of collagen synthesis contributes most

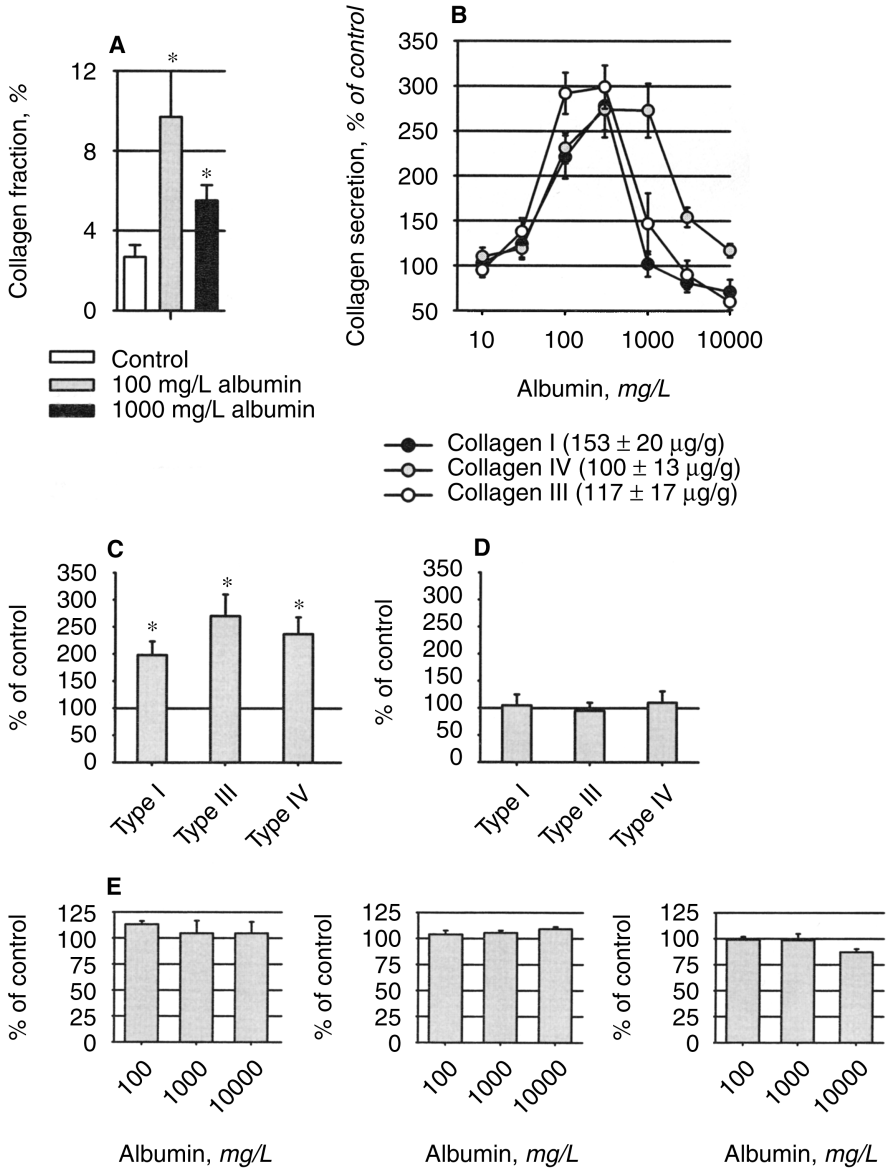

Fig. 1. Albumin-exposure enhances collagen secretion but not cell growth in OK cells. (*A*) Exposure to albumin for 48 hours enhances total collagen secretion as well as the collagen fraction in secreted protein ($N = 12–15$ for all data plotted). $*P < 0.05$ vs. control. (*B*) Collagen-ELISA shows that 48-hour exposure to albumin exerts a biphasic effect on the secretion of collagen I, III, and IV ($N = 30–45$ for all data plotted). The number in parentheses gives the rate of collagen secretion per 48 hours under control conditions. (*C*) Collagen secretion is also enhanced by 48-hour exposure to 10 mg/L γ-globulins, but not by exposure to 100 mg/L dextran (70 kD) (*D*) ($N = 3–9$ for all data plotted. (*E*) Cell number (left) or cell size (middle) or protein per cell (right) is not affected by exposure to albumin for 48 hours ($N = 15$ for all data plotted).

probably to the bisphasic dose-response curve shown in Fig. 1B.

Determination of gelatinase activity

In order to test whether reduced degradation of secreted collagen is involved in the above-described effects, we performed a gelatinase assay (Figs. 2 C and D). Exposure of OK cells to 100 mg/L of albumin for 48 hours led to a significant reduction of gelatinase activity in the media.

Determination of signaling pathway activation by reporter gene assay

Using a vector with the NF-κB-response element, exposure to albumin led to a clear dose-dependent increase of SEAP-activity (Fig. 3A). The response could be prevented by BIM (inhibition of PKC) or PDTC (inhibition of NF-κB-activation), but not by H89 (inhibition of PKA). Exposure of the cells to dextran was without effect. Virtually the same effects could be observed when the activator protein 1 (AP1)-response element was used (Fig. 3B). Exposure to albumin also stimulated the NFAT-pathway (i.e., PKC activation, Fig. 3C) and, to our surprise, the CRE-pathway (i.e., PKA activation, Fig. 3C). In agreement with previous studies [8], which showed ERK1/2-activation by albumin exposure, the SRE-pathway was also activated.

Signaling pathways involved in collagen secretion

We used different inhibitors for the above-mentioned pathways in order to test their contribution to collagen secretion. Fig. 3D summarizes the results. Because the

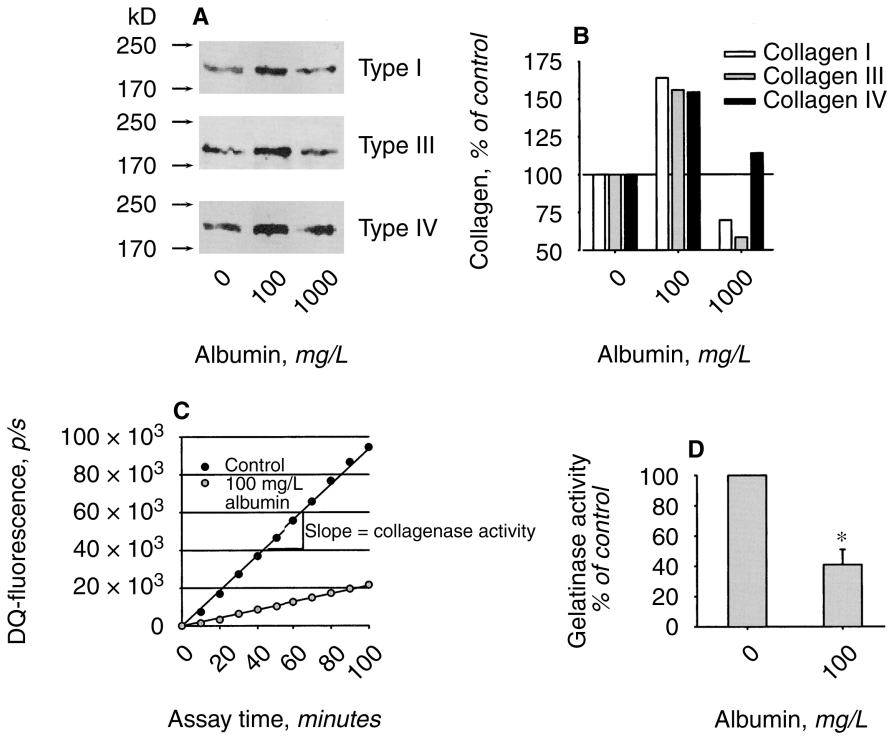

Fig. 2. Albumin-exposure enhances collagen synthesis and reduces collagen degradation. (*A*) Representative collagen Western blots with lysates from OK cells. (*B*) Densitometric analysis of the collagen Western blots (means of two experiments are shown). At 100 mg/L, albumin cellular collagen is increased. At 1000 mg/L, albumin collagen types I and III show a decrease below control. (*C*) Typical tracing of collagenase activity determination in media. In media from cells exposed to albumin for 48 hours, collagenase activity is reduced. Addition of albumin to the assay has no effect, thereby excluding artifacts. (*D*) Summary of data from several experiments ($N = 12$). $*P <$ 0.05 vs. the respective control.

inhibitors without albumin exposure exerted no significant effects, their data are not shown. Inhibition of PCK (BIM), NF-κB-activation (PDTC), or PKA (H89) significantly reduced albumin-induced secretion of collagens I, III, and IV. Inhibition of ERK1/2-activation (U0126) did not prevent collagen secretion, but even potentiated collagen I secretion.

Is protein uptake important for the observed effects?

We compared different cell types with different albumin endocytosis activity, and we inhibited albumin endocytosis in OK cells by 30 μmol/L EIPA. The rates of endocytosis and the rationale for using EIPA have been described extensively in previous studies [2, 11, 12]. In the two cell types (OK, LLC-PK_1), which express the megalin-cubilin scavenger receptor complex and show receptor-mediated protein endocytosis in a range close to the in vivo situation [6, 15], albumin enhanced collagen secretion (Fig. 4A). By contrast, in cells with low rates of protein endocytosis (NHE3-deficient OK cells, IHKE1, MDCK), exposure to albumin reduced collagen secretion below the control level. Inhibition of albumin endocytosis by EIPA (Fig. 4C, 30 μmol/L, a concentration that reduces albumin endocytosis by 70%) [11] exerted a significant effect on albumin-induced collagen secretion. Finally, we tested whether activation of signaling pathways by albumin exposure also depends on endocytosis, using NF-κB-activation as an example. Figs. 4B and D show that albumin-induced activation of the NF-κB-pathway also depends on endocytosis.

DISCUSSION

In the present study, we used cultured cells derived from the proximal tubule (not including MDCK cells) with different activities of receptor-mediated protein endocytosis in order to address the following three questions: (*1*) Do filtered proteins affect collagen homeostasis of proximal tubular cells; (*2*) if so, which signaling pathways are involved; and (*3*) is endocytic uptake of the proteins required? The two cell types, OK and LLC-PK_1, express the megalin-cubilin scavenger receptor complex and show receptor-mediated protein endocytosis in a range close to the proximal tubule [6, 15]. Therefore, these two cell types most closely resemble the situation in vivo.

Our data show that protein exposure, in a concentration range expected during enhanced protein filtration (≤1000 mg/L), leads to increased secretion of collagen I, III, and IV. Collagens I and III, especially, may contribute to tubulointerstitial fibrosis during chronic renal diseases, whereas collagen type IV may induce basement membrane thickening [16]. The underlying mechanisms involve enhanced synthesis and reduced degradation of secreted collagens. A similar mechanism has been described for the effects of glucose in mesangial cells [17], and hypoxia in proximal tubular cells [18]. Additional work is needed in order to determine whether the expression of matrix metalloproteases is reduced, or whether the expression of inhibitors of matrix metalloproteases (TIMPs) is enhanced.

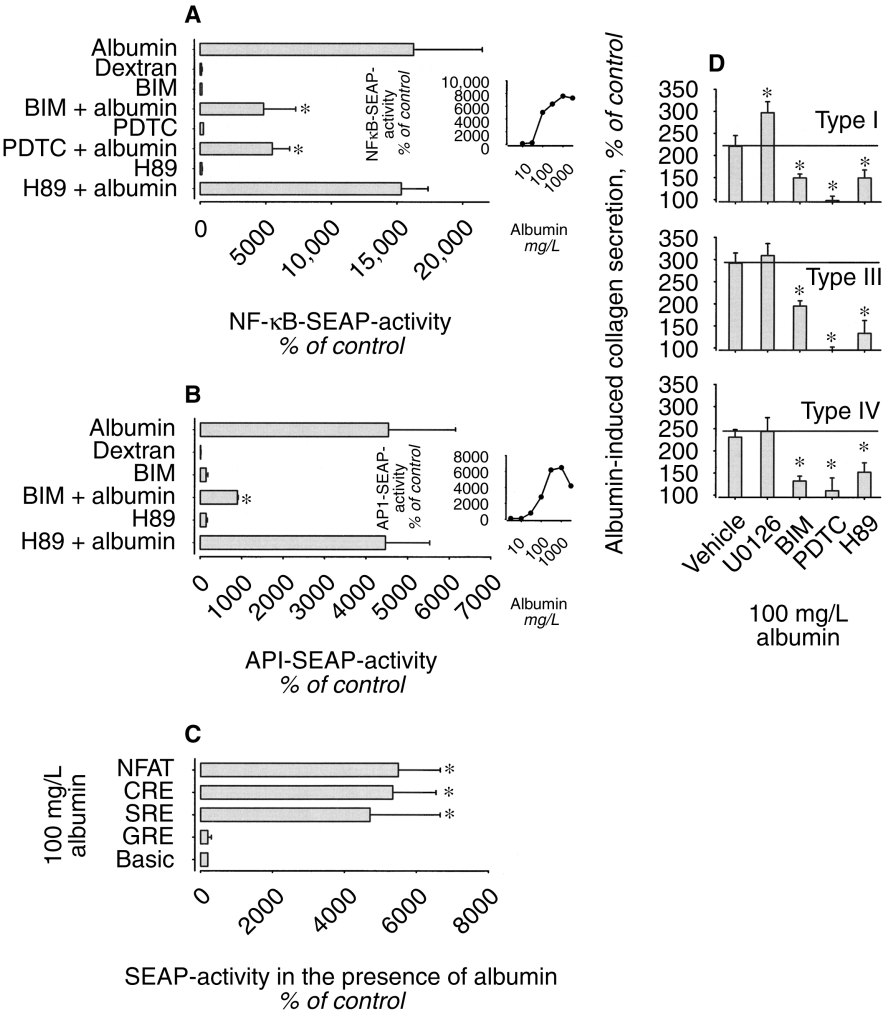

Fig. 3. Stimulation of signaling pathways during exposure to albumin. (*A*) Summary of the NF-κB-SEAP reporter assay data from several experiments. One hundred mg/L of albumin, 100 mg/L of dextran, 100 nmol/L BIM, 5 μmol/l H89, and 100 μmol/L PDTC were used ($N = 9$–15). *$P < 0.05$ vs. the respective control. All SEAP-data were corrected for transfection efficiency. The inset shows that the action of albumin is concentration dependent. Addition of albumin to the assay had no effect, thereby excluding artifacts. (*B*) Summary of the AP1-SEAP reporter assay data from several experiments. One hundred mg/L of albumin, 100 mg/L of dextran, 100 nmol/L BIM, and 5 μmol/L H89 were used ($N = 6$–12). *$P < 0.05$ vs. the respective control. All SEAP-data were corrected for transfection efficiency. The inset shows that the action of albumin is concentration dependent. (*C*) Summary of SEAP reporter assay data using different enhancer elements. The data show that albumin stimulates the NF-κB, AP1, NFAT, SRE (serum response element), and CRE (PKA) pathways, but not the GRE (glucocorticoid response element) pathway. In addition, no effect is observed with reporter plasmids without enhancer elements ($N = 6$–15). *$P < 0.05$ vs. control. (*D*) Albumin-induced collagen secretion (100 mg/L albumin, 48 hours) is reduced by 100 nmol/L BIM (inhibition of PKC), 5 μmol/L H89 (inhibition of PKA), or 100 μmol/L PDTC (inhibition of NF-κB-activation), but not by 10 μmol/L U0126 (inhibition of ERK1/2-activation) ($N = 6$–9). *$P <$ 0.05 vs. albumin alone. Addition of the inhibitors alone had no significant effect.

The signaling pathways activated by protein exposure include NF-κB, AP1, protein kinase C, and protein kinase A (Fig. 5). The involvement of NF-κB and AP1 is in good agreement with data from literature [19] which indicate that the simultaneous activation of different pathways is necessary for tubulointerstitial scarring during chronic renal diseases. Activation of PKC seems to be located upstream of NF-κB and AP1, because inhibition of PKC prevented the stimulation of NF-κB and AP1. Thus, one sequence of events contains activation of PKC, possibly by reactive oxygen species [20], followed by NF-κB-stimulation and subsequent increase in collagen secretion. PKA, which has been show to promote collagen synthesis also in cardiac fibroblasts [21], seems to act independently, because inhibition of PKA did not prevent NF-κB and AP1 stimulation, but reduced albumin-induced collagen secretion. Further studies will be necessary to precisely determine the crosstalk between the different signaling pathways. Of course, we have to consider the possibility that endocytosed albumin does not stimulate the above mentioned signaling pathways directly, but via an autocrine activation loop after secretion of TGFβ1 (Fig. 5). In rats, it has been shown that albuminuria is linked to enhanced renal TGFβ1 expression [22].

Finally, our data strongly indicate that the mere presence of proteins in the proximal tubule is not sufficient for either signaling pathway activation or collagen secretion. There has to be efficient receptor-mediated endocytosis of the proteins, most probably by the megalin-cubilin-complex, in order to stimulate collagen secretion. Sole increases in the extracellular albumin concentration inhibit collagen secretion by yet unknown mechanisms. Possibly, high extracellular albumin concentrations act as radical scavengers and protect tubular cells [23]. The protective effects of extracellular proteins can also explain the biphasic action of albumin in OK cells, where high albumin concentrations (>1000 mg/L) reduced collagen secretion, at least in part. In addition, according to these data, the use of pathophysiologically-relevant concentrations of albumin and proximal tubular cells

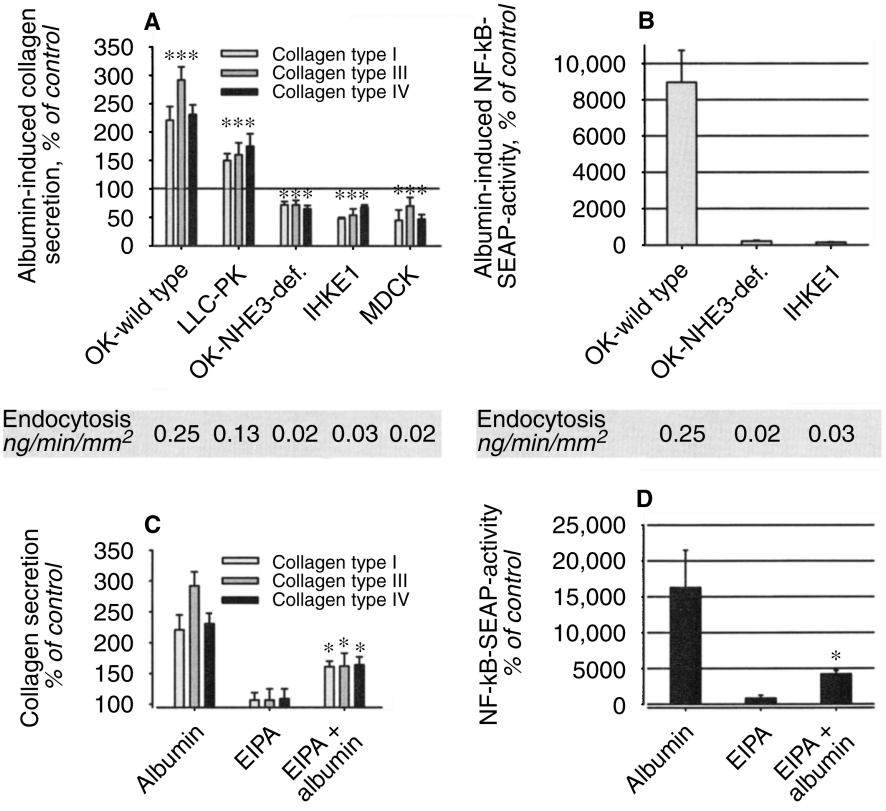

Fig. 4. Albumin endocytosis is necessary for albumin-induced collagen secretion. (*A*) Albumin-induced collagen secretion (100 mg/L albumin, 48 hours) in cell types with different endocytic activity. The rate of endocytosis in the proximal tubule is ~0.45 ng albumin/min/mm^2. Only cells with high rates of endocytosis show albumin-induced collagen secretion (N = 6–15 for all values plotted). *$P < 0.05$ vs. control. (*B*) Albumin-induced stimulation of the NF-κB-pathway is observed only in cell types with high rate of endocytosis (N = 6–15). (*C*) Inhibition of albumin endocytosis in OK cells with 30 μmol/L EIPA (in this case, inhibition of apical NHE3) reduces albumin-induced collagen secretion significantly (N = 6). *$P < 0.05$ vs. control (100 mg/L albumin, 48 hours). (*D*) Albumin-induced stimulation of the NF-κB-pathway is reduced when endocytosis is inhibited by 30 μmol/L EIPA (N = 9). *$P < 0.05$ vs. albumin alone. Abbreviations are: OK-NHE3-def, OK-cell deficient for NHE3; IHKE1, immortalized human kidney epithelial cells; MDCK, Madin-Darby canine kidney cells.

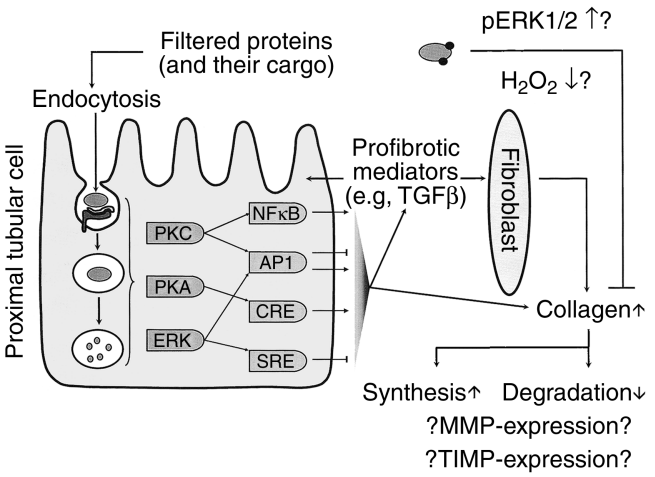

Fig. 5. Hypothetical model summarizing the data from this study. When the filtration of proteins (and their cargo) increases, the amount of protein taken up by receptor-mediated endocytosis in the proximal tubule also increases, leading to the stimulation of different signaling pathways. Stimulation of these pathways induces enhanced secretion of collagen I, III, and IV due to enhanced collagen synthesis and reduced collagen degradation. At present, it is not clear whether the rate of MMP expression is reduced or the expression of inhibitors is increased. Possibly, an autocrine activation loop, via TGF-β1, contributes to the observed effects. The increase of extracellular protein is not sufficient in order to enhance collagen secretion. On the contrary, when extracellular albumin increases without being taken up efficiently by the cells, it leads to a reduction of collagen secretion.

with a reasonable endocytic activity is required for studies on proteinuria-induced tubulointerstitial damage.

In summary, the data show that endocytic uptake of proteins disturbs collagen homeostasis in proximal tubular cells. This disturbed matrix homeostasis probably supports the progression of interstitial fibrosis, which is important for the development of renal insufficiency. Intervention with protein endocytosis may represent a therapeutic strategy.

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